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## TRE17/USP6 oncogene translocated in aneurysmal bone cyst induces matrix metalloproteinase production via activation of NFκB

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### Abstract

Aneurysmal bone cyst (ABC) is an aggressive, pediatric bone tumor characterized by extensive destruction of the surrounding bone. Though first described over 60 years ago, its molecular etiology remains poorly understood. Recent work revealed that ABCs harbor translocation of *TRE17/USP6*, leading to its transcriptional upregulation. TRE17 encodes a ubiquitin-specific protease (USP), and a TBC domain that mediates binding to the Arf6 GTPase. However, the mechanisms by which TRE17 overexpression contributes to tumor pathogenesis, and the role of its USP and TBC domains are unknown. ABCs are characterized by osteolysis, inflammatory recruitment, and extensive vascularization, processes in which matrix proteases play a prominent role. This led us to explore whether TRE17 regulates the production of matrix metalloproteinases (MMPs). In the current study, we demonstrate that TRE17 is sufficient to induce expression of MMP-9 and MMP-10, in a manner requiring its USP activity, but not its ability to bind Arf6. TRE17 induces transcription of MMP-9 through activation of NFκB, mediated in part by the GTPase RhoA and its effector kinase, ROCK. Furthermore, xenograft studies demonstrate that TRE17 induces formation of tumors that reproduce multiple features of ABC, including a high degree of vascularization, with an essential role for the USP domain. In sum, these studies reveal that TRE17 is sufficient to initiate tumorigenesis, identify MMPs as novel TRE17 effectors that likely contribute to ABC pathogenesis, and define the underlying signaling mechanism of their induction.

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## Keywords

TRE17/USP6; aneurysmal bone cyst; MMP-9; Arf6; NF $\kappa$ B; RhoA

## INTRODUCTION

Ubiquitin-specific proteases (USPs) constitute a large subfamily of de-ubiquitinating enzymes that function in diverse cellular processes, including the DNA damage response, inflammation, and oncogenesis (Amerik and Hochstrasser, 2004; Singhal *et al.*, 2008; Komander *et al.*, 2009). Several USPs have been implicated in human tumorigenesis. HAUSP/USP7 is a key regulator of the p53 tumor suppressor (Li *et al.*, 2002). HAUSP de-ubiquitinates p53 to rescue it from proteasomal degradation. CYLD encodes a tumor suppressor that is mutated in familial cylindromatosis, an autosomal dominant disease characterized by benign tumors arising from cells of skin appendages. CYLD is a negative regulator of the transcription factor nuclear factor- $\kappa$ B (NF $\kappa$ B), and functions by de-ubiquitinating the ubiquitin ligases TRAF-2 and TRAF-6 (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003).

Another USP strongly implicated in human neoplasia is *TRE17/USP6*, which is translocated at high frequency in aneurysmal bone cyst (ABC), a pediatric osseous neoplasm (Cottalorda and Bourelle, 2007; Saccomanni, 2008). Though benign, ABCs cause extensive bone degradation and impinge on surrounding tissue due to their expansile nature, causing pain, swelling, deformity, neurological symptoms, and pathologic fracture (Cottalorda and Bourelle, 2007; Saccomanni, 2008). Though first described over 60 years ago, the etiology of ABC has remained poorly understood. They were long thought to be reactive lesions, arising from local vascular disturbances. However, recent work showed that translocation of *TRE17* occurs in over 60% of ABC cases, revealing their neoplastic origin (Panoutsakopoulous *et al.*, 1999; Althof *et al.*, 2004; Oliveira *et al.*, 2004a; Oliveira *et al.*, 2004b; Oliveira *et al.*, 2005; Sukov *et al.*, 2008). Five different fusion partners of *TRE17* have been identified, and in every case translocation resulted in promoter swapping, where the complete coding sequence of *TRE17* was placed downstream of the highly active promoter of its fusion partner, leading to its transcriptional upregulation (Oliveira *et al.*, 2004a; Oliveira *et al.*, 2004b; Oliveira *et al.*, 2005). Notably, *TRE17* is absent or expressed at very low levels in most adult human tissues except for testes (Paulding *et al.*, 2003).

These studies strongly suggest that *TRE17* overexpression plays a key role in the etiology of ABC. Histologically, ABCs are complex lesions consisting of blood-filled cysts, separated by fibrous stromal areas containing spindle cells, inflammatory cells, and numerous capillaries. Strikingly, *TRE17* translocation occurs exclusively in spindle cells within the fibrous stroma that are believed to represent an early phase of osteoblast differentiation (Oliveira *et al.*, 2004b). This is supported by the fact that the promoters of *TRE17*'s fusion partners are highly active during osteoblast differentiation and bone formation (Oliveira *et al.*, 2004a; Oliveira *et al.*, 2004b; Oliveira *et al.*, 2005). Indeed, one fusion partner, *OMD*, is expressed selectively in osteoblasts. Furthermore, osteoid (the organic component of bone matrix produced by osteoblasts) is commonly present in these lesions. Together, these observations suggest that cells overexpressing *TRE17* in ABCs are immature cells of the osteoblast lineage. However, the mechanism by which overexpression of *TRE17* in osteoblasts contributes to the formation of these complex, cellularly heterogeneous lesions is completely unknown.

Two naturally occurring splice variants of *TRE17* exist (Nakamura *et al.*, 1992; Paulding *et al.*, 2003), giving rise to isoforms termed *TRE17*(long) and *TRE17*(short) (previously

termed TRE17(onco)). They are identical through the first 773 amino acids, but then diverge: TRE17(long) encodes a USP, which comprises cysteine and histidine subdomains, both of which are required for catalytic activity (Papa and Hochstrasser, 1993). TRE17(short) is truncated shortly after the cysteine subdomain. Both isoforms contain an N-terminal TBC (TRE2-Bub2-Cdc16) domain. TBC domains are generally predicted to encode GTPase-activating proteins (GAPs) for Rab family GTPases (Bernards, 2003). However, we and others have shown that the TBC domain of TRE17 lacks catalytic activity, and that it functions instead to mediate interaction with the small GTPase, Arf6 (Martinu *et al.*, 2004; Bizimungu and Vandenbol, 2005; Bizimungu *et al.*, 2007). We further reported that TRE17 promotes activation of Arf6 *in vivo* (Martinu *et al.*, 2004). However, the mechanism by which TRE17 contributes to ABC pathogenesis, and the roles of its TBC and USP domains, remain largely unknown. Here, we identify matrix metalloproteinases as novel effectors of TRE17, and dissect the signaling pathways leading to their induction. We further demonstrate that TRE17 is sufficient to induce the formation of tumors that recapitulate multiple features of ABC, in a manner that requires its USP domain.

## RESULTS

### TRE17 induces production of MMP-9 and MMP-10 in a USP-dependent manner

ABCs are characterized by extensive bone degradation, inflammatory recruitment, and angiogenesis, processes in which matrix proteases play a key role. Furthermore, a previous immunohistochemical study showed that the fibrous stromal regions of ABC stain strongly for matrix metalloproteinase-9 (MMP-9) (Kumta *et al.*, 2003). This led us to explore whether TRE17 contributes to ABC pathogenesis by regulating production of MMPs. Using an unbiased antibody-based array to screen the conditioned medium (CM) of transfected cells, we found that TRE17 induced the accumulation of MMP-9 and MMP-10 (Supplemental Figure 1). These array results were confirmed by direct immunoblotting, performing studies in osteoblastic cells, the lineage that harbors translocation/overexpression of TRE17 in ABC. As shown in Figure 1A, transient expression of TRE17(long) in hFOB1.19, a human pre-osteoblastic cell line (Subramaniam *et al.*, 2002), induced production of MMP-9 and MMP-10, as determined by immunoblotting of CM from serum-starved cells.

We next examined the roles of TRE17's TBC and USP domains in MMP induction. To determine the requirement for TRE17's USP activity, we tested the naturally occurring TRE17(short) isoform, as well as a catalytically inactive point mutant (TRE17(long)/USP-) (Shen *et al.*, 2005) (Figure 1B). As seen in Figure 1A, both of these alleles failed to induce MMP production in hFOB1.19 cells. We also assessed the role of the TBC domain, which we previously reported mediates binding to Arf6 (Martinu *et al.*, 2004). We generated a triple point mutant within the TBC domain that ablates binding to Arf6 (denoted TRE17(A6-)) (Figure 1B and 1C). This mutant was competent to induce MMP production as well as TRE17(long) (Figure 1A). Together, these data indicate that TRE17's USP activity, but not its ability to bind Arf6, is required for the induction of MMP- and MMP-10.

We wished to confirm these results in an independent osteoblastic system, under conditions of stable expression. For this purpose, MC3T3-E1 murine pre-osteoblasts (Wang *et al.*, 1999), referred to hereafter as MC3T3, were engineered to stably express TRE17 alleles in a doxycycline (dox)-inducible manner. Figure 1D demonstrates uniform expression of the various mutants, strictly in the presence of dox. MC3T3 cell lines expressing TRE17(short) were also generated, however its expression levels were significantly reduced relative to the other alleles (data not shown). Therefore, cells expressing TRE17(long)/USP- were used in subsequent experiments to examine the role of the USP domain. Dox-dependent induction of MMP-9 was observed in the MC3T3 lines expressing TRE17(long) and TRE17(A6-), as

determined by both immunoblotting and zymography, which measures gelatinolytic activity (Figure 1D). In contrast, TRE17(long)/USP<sup>-</sup> was significantly impaired in stimulating MMP-9 production (Figure 1D). Effects on MMP-10 could not be confirmed in these cells by immunoblotting, since antibodies that recognize the murine protein are not available. However, alternative detection methods revealed that it was also induced (see next section).

We next explored whether MMP induction by TRE17 was specific to osteoblasts, or could be elicited in other cell types. To test this, TRE17 alleles were transfected into HeLa cells, a cervical carcinoma. As seen in Figure 1E, TRE17(long) was capable of inducing robust expression of MMP-9 and MMP-10 in these cells, again in a USP-dependent manner. Thus, TRE17's ability to stimulate matrix protease production is not restricted to osteoblasts. As mentioned above, previous work showed that ABC tumors stain strongly for MMP-9 (Kumta *et al.*, 2003), but the cells responsible for its production had not been identified. Our data demonstrate that TRE17 is sufficient, in the absence of any serum-derived factors, to induce production of MMPs, and suggest that it may be directly responsible for their production in ABCs.

### TRE17 stimulates MMP expression on a transcriptional level

We next sought to determine the mechanism by which TRE17 induces production of MMPs. Because transcriptional induction is the most common mode of MMP regulation, (Sternlicht and Werb, 2001; Mott and Werb, 2004), we examined whether TRE17 increased the abundance of MMP mRNA. RNA was isolated from transfected hFOB1.19 or HeLa, or the MC3T3 stable lines. RT-quantitative PCR was performed using two independent primer pairs for each MMP, and normalized against GAPDH. In all three systems, levels of MMP-9 and MMP-10 mRNA were elevated in cells expressing TRE17(long), but not TRE17(short) or TRE17(long)/USP<sup>-</sup> (Figure 2A-C).

We next tested whether increased MMP expression resulted from transcriptional induction. We focused on MMP-9 since its expression in ABC had been previously demonstrated, and there exists a wealth of information on its regulation as well as reagents for its analysis. Reporter assays were performed using a luciferase construct driven by base pairs -674 to +3 of the human MMP-9 promoter (Liu *et al.*, 2006). In both hFOB1.19 and HeLa, TRE17(long) activated the MMP-9 promoter approximately 5-fold over control vector transfected samples (Figure 2D). TRE17(short) and TRE17(long)/USP<sup>-</sup> were significantly compromised in activation of the reporter. Transient reporter assays could not be performed in the MC3T3 cell lines as they were engineered to stably and inducibly express luciferase. Nevertheless, these data in sum reveal that TRE17 induces transcription of MMP-9 in a USP-dependent manner.

### NFκB is required for induction of MMP-9 by TRE17

Two key regulators of the MMP-9 promoter are NFκB (Liu *et al.*, 2006) and AP-1 (Cho *et al.*, 2000; Genersch *et al.*, 2000; Kim and Lee, 2005; Han *et al.*, 2006). To ascertain their roles in MMP-9 induction, we first analyzed a reporter construct mutated in the NFκB response element (Liu *et al.*, 2006). Loss of the NFκB-binding site abrogated activation of the MMP-9 promoter by TRE17(long) in hFOB1.19 and HeLa (Figure 3A), indicating that NFκB is the key mediator of TRE17-induced MMP-9 gene transcription.

To confirm the dominant role of NFκB in induction of endogenous MMP-9 by TRE17, we examined the effects of inhibitors of the NFκB and AP-1 pathways on MMP-9 secretion. NFκB activation was blocked using Bay11-7082 (Pierce *et al.*, 1997). Bay11-7082 caused a dose-dependent reduction in levels of MMP-9 induced by TRE17(long), in hFOB1.19 and MC3T3 cells (Figure 3B). However, U0126, which inhibits AP-1 activation by preventing



its phosphorylation by Erk, had no effect on MMP-9 secretion (Figure 3B). To confirm the requirement for NF $\kappa$ B, we tested the effects of three additional structurally unrelated NF $\kappa$ B pathway inhibitors, wedelolactone, SC-514, and IKK Inhibitor XI. As shown in Figure 3C, all three drugs significantly inhibited MMP-9 production in both HeLa and MC3T3 cells. Together, these results confirm that induction of MMP-9 by TRE17(long) is predominantly mediated through NF $\kappa$ B.

### TRE17 activates NF $\kappa$ B in a USP-dependent manner

The requirement for NF $\kappa$ B in MMP-9 production led us to explore whether TRE17 activates NF $\kappa$ B. As shown in Figure 4A, TRE17(long) potently activated an NF $\kappa$ B-responsive luciferase reporter in both hFOB1.19 and HeLa, while TRE17(short) and TRE17(long)/USP<sup>-</sup> were attenuated by comparison. Activation of NF $\kappa$ B by TRE17(long) was confirmed by electrophoretic mobility shift assays (EMSA) (Figure 4B), and by monitoring its subcellular localization (Figure 4C). NF $\kappa$ B, which comprises p65 and p50 subunits, is maintained in an inactive state through its cytoplasmic retention by I $\kappa$ B; activation is reflected by nuclear translocation of p65/p50. In control hFOB1.19, p65 exhibited diffuse cytoplasmic staining and was largely excluded from the nucleus (Figure 4C). In contrast, in cells expressing TRE17(long), but not TRE17(short) or TRE17(long)/USP<sup>-</sup>, p65 accumulated in the nucleus (Figure 4C). Similar results were seen in HeLa cells and in the MC3T3 cell lines (data not shown). Together, these data confirm that TRE17 activates NF $\kappa$ B in a USP-dependent manner.

### RhoA/ROCK mediate NF $\kappa$ B activation and MMP-9 production by TRE17

We next explored the signaling mechanism by which TRE17 induces activation of NF $\kappa$ B. NF $\kappa$ B can be activated by a wide variety of signaling molecules, including Rho family GTPases. During the course of our studies, we noted that cells expressing TRE17 exhibited alterations in morphology that were consistent with activation of RhoA (Nobes and Hall, 1995a; Nobes and Hall, 1995b). Specifically, they had a greatly increased number of focal adhesions distributed throughout the cell (data not shown). To determine whether RhoA was activated by TRE17, we performed pulldown assays using the Rho-binding domain (RBD) of Rhotekin (which binds to RhoA in a GTP-dependent manner) as an affinity reagent. Expression of TRE17(long) in hFOB1.19 or HeLa induced robust activation of RhoA (Figure 5A). Activation of RhoA was also observed in TRE17(long)/MC3T3, in a dox- and USP-dependent manner (Figure 5A).

Previous work had demonstrated that RhoA potently activates NF $\kappa$ B, functioning through its effector kinase, ROCK (Benitah *et al.*, 2003; Anwar *et al.*, 2004; Nakakuki *et al.*, 2005; Cui *et al.*, 2006). This led us to investigate whether RhoA and ROCK mediate TRE17-induced activation of NF $\kappa$ B. Co-expression of dominant negative RhoA partially attenuated activation of the NF $\kappa$ B-luc reporter by TRE17(long), in both hFOB1.19 and HeLa cells (Figure 5B). Furthermore, treatment of cells with the ROCK inhibitor Y27632 also partially inhibited NF $\kappa$ B activation by TRE17(long), in a dose-dependent manner (Figure 5B).

Since we showed that NF $\kappa$ B is essential for induction of MMP-9, we predicted that Y27632 should also block MMP-9 production. Accordingly, we found that Y27632 caused a dose-dependent reduction in MMP-9 levels induced by TRE17(long)/MC3T3, as determined by immunoblotting and zymography (Figure 5C). Collectively, our data define a pathway in which TRE17, in a USP-dependent manner, activates RhoA/ROCK, leading to NF $\kappa$ B activation and resultant production of MMP-9.

## TRE17 induces formation of tumors that recapitulate multiple features of ABC in a USP-dependent manner

In sum, our *in vitro* analysis demonstrates that TRE17 is sufficient to induce expression of MMPs, which we propose contributes to ABC pathogenesis. MMPs (MMP-9 in particular) play a central role in angiogenesis and inflammation, processes which typify ABC lesions. This led us to examine whether xenografts of TRE17(long)-expressing MC3T3 recapitulate molecular features of this tumor. We elected to introduce cells into mice subcutaneously rather than intra-osseously in order to facilitate both delivery and monitoring of the xenograft. Subcutaneous placement of the xenograft is physiologically relevant since ABCs can arise in soft tissues, and are histologically identical to osseous cases (Nielsen *et al.*, 2002; Sukov *et al.*, 2008).

MC3T3-derived lines were injected subcutaneously into flanks of nude mice, and expression of TRE17(long) and luciferase was induced *in vivo* by addition of dox to the drinking water. Palpable growths were visible as early as 5-7 days post-injection in all 9 mice injected with TRE17(long)/MC3T3, exclusively in the presence of dox. Tumors reached maximal size (from 3-10 mm in diameter) within 2 weeks. No palpable tumors were observed in any mice injected with MC3T3 expressing TRE17(long)/USP-, TRE17(short), or luciferase alone.

To assess tumorigenesis quantitatively, *in vivo* bioluminescence imaging was performed. Animals were injected intraperitoneally with luciferin, then imaged using the Xenogen In Vivo Imaging System to detect the luciferase-expressing xenografted cells. While all animals injected with TRE17(long)/MC3T3 cells emitted a strong bioluminescence signal, mice injected with cells expressing TRE17(long)/USP-, TRE17(short), or luciferase alone gave signals approximately 10-fold weaker (Figure 6A and data not shown). These results reveal that TRE17's USP activity is essential for tumorigenesis.

Dissection of animals injected with TRE17(long)/MC3T3 cells revealed the presence of solid tumors with a striking hemorrhagic appearance (Figure 6B). Hematoxylin and eosin staining revealed that this arose from extensive vascularization as well as blood extravasation (Figure 6B), both of which are prominent features of ABC. Lesions were composed of a spindle cell population associated with a fibrous or myxoid matrix (Fig 6B), also highly reminiscent of ABC (Oliveira *et al.*, 2004b; Shinde *et al.*, 2006). No tumors or vascularization were seen upon dissection of mice injected with control or TRE17(long)/USP<sup>-</sup>-expressing MC3T3 cells (Figure 6B). Immunohistochemical analysis of serial tumor sections revealed strong MMP-9 staining of the TRE17-positive cells (Figure 6C). These results not only provide *in vivo* confirmation of our *in vitro* results, but also recapitulate the MMP-9 enrichment observed in the stroma of ABC tumors (Kumta *et al.*, 2003). Thus, our data demonstrate that this osteoblastic model reproduces multiple features of ABC, and strongly validate its use for dissecting the molecular functions of TRE17 in ABC pathogenesis.

## DISCUSSION

Although aneurysmal bone cyst was first described over 60 years ago, remarkably little progress has been made in elucidating its pathogenesis. The discovery in 2004 that translocation of *TRE17/USP6* occurs in the majority of ABC cases represented a significant advance. However, many key questions have remained unanswered regarding TRE17's role in the etiology of this tumor, including whether TRE17 overexpression is sufficient to initiate tumor formation, the molecular pathways it engages, and the role of its USP domain.

Our study provides insights into all of these key issues. We show that overexpression of TRE17 in pre-osteoblastic MC3T3 cells is sufficient to drive the formation of tumors that

reproduce molecular and histological features of ABC. Interestingly, TRE17(long) tumors remained as solid masses, and did not form the multicystic lesions typically seen in ABC. Thus, although TRE17 is sufficient to initiate tumor formation, additional events may be required to produce the full spectrum of characteristics of these complex lesions. Alternatively, the MC3T3 system might not precisely mimic the cellular context of the TRE17-translocated cells in ABC. Nevertheless, our results refute the prevailing model for the pathogenesis of ABC, which posits that it originates from a local circulatory disturbance, with resultant tissue damage. Our data instead suggest a direct role for TRE17 in establishing a degradative and vascularized microenvironment.

This work further identifies MMPs as novel effectors of TRE17. Previous immunohistochemistry showed that MMP-9 is expressed in the fibrous stroma of ABC, but the cells responsible for its production had not been determined (Kumta *et al.*, 2003). Furthermore, it was unclear whether MMP-9 had a causal role in ABC pathogenesis, or was produced secondarily as a result of tissue damage and inflammation. Our finding that TRE17 directly induces its production supports the former scenario, and suggests that MMP-9 is a relevant effector of TRE17 *in vivo*.

There are multiple means by which MMP-9 and MMP-10 could contribute to ABC pathogenesis. Both of these MMPs have previously been implicated in remodeling/degradation of bone matrix, under physiological and pathological conditions (Bord *et al.*, 1998; Giambernardi *et al.*, 1998; Engsig *et al.*, 2000; Sternlicht and Werb, 2001; Onodera *et al.*, 2002; Tolboom *et al.*, 2002; Kumta *et al.*, 2003; Haeusler *et al.*, 2005; Barksby *et al.*, 2006; Kanangat *et al.*, 2006). MMP-9 and MMP-10 have multiple substrates including other MMPs, which induces their zymogenic activation (Sternlicht and Werb, 2001; Van den Steen *et al.*, 2002; Krampert *et al.*, 2004), potentially further contributing to the degradative microenvironment in ABC. They can also proteolyze components of the ECM, which serves not only to degrade the ECM but also to liberate matrix-bound growth factors that further promote inflammation and angiogenesis (Sternlicht and Werb, 2001). For example, numerous studies indicate that MMP-9 plays an important role in release of VEGF from bone and cartilage matrix to promote angiogenesis and vascular permeability (Vu *et al.*, 1998; John and Tuszynski, 2001; Rundhaug, 2005; Ghajar *et al.*, 2008). MMP-9 has also been proposed to contribute to angiogenesis by degrading the endothelial and interstitial matrix to facilitate endothelial cell migration and branching. Thus, the known functions of MMP-9 are fully consistent with the histological features of ABC, which contain numerous capillaries and extravasated blood. Future studies aimed at blocking MMP-9 expression/activity will determine whether it is indeed required for the extensive vascularization observed in our xenograft model.

Our studies further dissect the mechanism by which TRE17 induces MMP expression. Induction of MMP-9 and MMP-10 is dependent on TRE17's USP activity, but not its ability to bind Arf6. Furthermore, NF $\kappa$ B is required for transcriptional induction of MMP-9. Activation of NF $\kappa$ B is also dependent on TRE17's USP activity, and is partially mediated through RhoA/ROCK. The substrate(s) of TRE17's USP domain and the mechanism by which it leads to activation of RhoA remain to be determined. Previous work showed that RhoA can be ubiquitinated to trigger its proteasomal degradation (Wang *et al.*, 2003; Bryan *et al.*, 2005; Wang *et al.*, 2006). However, it is unlikely that TRE17 functions by de-ubiquitinating and stabilizing RhoA, since total RhoA levels are unaffected by TRE17. A more likely possibility is that TRE17's USP domain targets a guanine nucleotide exchange factor (GEF) for RhoA to rescue it from proteasomal degradation.

Since inhibition of RhoA/ROCK only partially attenuated activation of NF $\kappa$ B by TRE17, this suggests that TRE17 functions through additional parallel pathways, in a manner that



requires its USP activity. One possibility is that it might de-ubiquitinate a component of the NF $\kappa$ B regulatory pathway. NF $\kappa$ B activation is exquisitely controlled by a complex array of ubiquitination and phosphorylation events (Hacker and Karin, 2006; Sebban *et al.*, 2006). Notably, three other USPs, CYLD, A20, and USP11, have previously been linked to NF $\kappa$ B regulation (Brummelkamp *et al.*, 2003; Kovalenko *et al.*, 2003; Trompouki *et al.*, 2003; Wertz *et al.*, 2004; Yamaguchi *et al.*, 2007). However, in contrast to TRE17, they function as negative regulators of NF $\kappa$ B, suggesting that TRE17 targets a distinct substrate from these other USPs.

In conclusion, this work defines a signaling pathway, from TRE17->RhoA/ROCK->NF $\kappa$ B->MMP-9, which we propose contributes to ABC pathogenesis. Our results demonstrate that TRE17's USP activity and NF $\kappa$ B are required for MMP-9 induction, raising the possibility that development of USP-specific inhibitors or NF $\kappa$ B antagonists might be effective novel strategies for the treatment of these tumors.

## MATERIALS AND METHODS

### Cell culture

HeLa were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin and streptomycin (P/S), sodium pyruvate and GlutaMax (Invitrogen). The murine pre-osteoblast cell line MC-3T3-E1 (Clone 14) (referred to hereafter as MC3T3) was obtained from ATCC, and grown in  $\alpha$ -Modified Eagle Media ( $\alpha$ -MEM) with tetracyclin-free FBS, P/S, sodium pyruvate, GlutaMax, and G418 (400  $\mu$ g/ml). Both lines were maintained at 37°C in 5% CO<sub>2</sub>. Human fetal osteoblast hFOB1.19 cells were grown in 1:1 DMEM without phenol red: Ham's F12, with the same supplements and G418 (300  $\mu$ g/ml active), and kept at 34 °C in 5% CO<sub>2</sub>.

### Antibodies and reagents

Antibodies for HA, actin, p65, and RhoA were from Santa Cruz Biotechnology, Inc. Anti-human MMP-9 (AF911), anti-mouse MMP-9 (MAB9091), and anti-human MMP-10 (MAB9101) were from R&D systems. Gelatin agarose was from Sigma. TRE17 was detected using anti-HA, or an antibody generated against the N-terminus of TRE17. Y27632, Bay11-7082, wedelolactone, SC514, and IKK Inhibitor XI (PS1145) were from EMD Biosciences.

### Generation of MC3T3 cell lines

TRE17 cDNAs (TRE17(long), TRE17(short), TRE17(long)/USP-, and TRE17(A6-) were subcloned into pBI-L (Clontech), which contains a bi-directional tet/dox-inducible promoter that drives simultaneous expression of TRE17 and firefly luciferase. Resulting plasmids were AseI-digested, then ligated to AseI-digested pN1 $\beta$ actin-rtTA2S-IRES-EGFP (Welman *et al.*, 2006). As negative control, pBI-L encoding luciferase alone was ligated to pN1 $\beta$ actin-rtTA2S-M2-IRES-GFP to generate pSMV (super module vector) (Welman *et al.*, 2006). MC3T3 cells were transfected with the pSMV-derived constructs, then selected in medium containing tet-free FBS and G418 (400  $\mu$ g/ml). GFP-positive cells were isolated from the G418-resistant pool by flow cytometry.

### Detection of MMPs from Conditioned Medium

Cells were starved under serum-free conditions for 24-48 hours prior to harvesting. Conditioned medium (CM) was subjected to low speed centrifugation to remove cell debris. To monitor MMP-9, CM was supplemented with protease inhibitors, incubated with gelatin agarose beads (Sigma) at 4°C for 6 hr, washed three times in PBS, then subjected to immunoblotting. For zymography assays, gelatin agarose purified MMP-9 was fractionated

on 8% gels containing 30 mg/ml gelatin (Sigma). Gels were washed with 2.5% Triton X-100, incubated in substrate buffer (50mM Tris-HCl pH 8.0, 5mM CaCl<sub>2</sub>) at 37°C for 36 hr, then stained with Coomassie Brilliant Blue. To detect MMP-10, CM was precipitated with 10% trichloroacetic acid. Pellets were washed twice in cold acetone, air dried, then fractionated by SDS-PAGE.

### Reverse transcription-quantitative PCR

RNA was extracted with Trizol (Invitrogen). Reverse transcription reactions were performed on 1-2 µg of total RNA using a Superscript First-Strand Synthesis System kit (Invitrogen). Real time PCR was performed using TaqMan probes on an ABI Prism 7900 system. Relative expression levels were determined from a standard curve of serial dilutions of cDNA samples and normalized to GAPDH levels. Primer sequences are available upon request.

### Luciferase Assays and EMSA

NFκB-dependent pBIIx (Zhong *et al.*, 1997) and human MMP-9 promoter (Liu *et al.*, 2006) reporter constructs have been previously described. β-actin promoter-driven *Renilla* luciferase reporter was used for normalization. HA-tagged TRE17 constructs were described previously (Masuda-Robens *et al.*, 2003; Martinu *et al.*, 2004; Shen *et al.*, 2005). Cells were seeded in 35 mm wells, transfected with Lipofectamine 2000 or FuGENE6, starved, then treated with drugs as indicated. Dual luciferase assays were performed according to manufacturer instructions (Promega). Evaluations of statistical significance were conducted with student's t test. A two-tail p value < 0.05 was considered statistically significant. EMSA was performed as previously described (Solt *et al.*, 2007).

### Immunofluorescence microscopy

Cells were seeded on coverslips, transfected using FuGENE6, then treated as described previously (Masuda-Robens *et al.*, 2003). Samples were viewed on a Zeiss confocal microscope with LSM510 software, using excitation wavelengths of 488 nm (FITC), 546 nm (Cy3), or 358 nm (DAPI).

### RhoA activity and TRE17-Arf6 binding assays

For RhoA activity assays, cells were lysed in RBD buffer (20 mM HEPES pH 7.5, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, pepstatin, leupeptin and aprotinin). Extracts were precipitated with 20 µg of glutathione-S-transferase (GST) fused to the Rho binding domain of Rhotekin conjugated to agarose for 2 hr, washed three times in RBD buffer, then immunoblotted with RhoA antibody. Association of TRE17 and Arf6 was monitored as described (Martinu *et al.*, 2004).

### Xenografting, Bioluminescence Imaging, and histology

All mouse procedures were in accordance with University of Pennsylvania IACUC. Nude mice (Jackson Laboratories) were fed water with dox (1 mg/ml containing 5% sucrose) for 1-2 weeks before xenografting. MC3T3 cell lines were pre-treated with dox one day before subcutaneous injection (2.5E6 cells per site). For dox-treated animals, 9 mice were injected per cell line; dox was maintained in water for duration of the experiment. For animals not treated with dox, 3 mice were injected per cell line.

For bioluminescence imaging, mice were anaesthetized with Avertin. Animals were injected intraperitoneally with D-luciferin (Biotium; 120 µl at 25 mg/ml), and imaged within 8-20 minutes using the Xenogen In Vivo Imaging System. Tumors were paraffin-embedded and

histological analysis was performed by the Abramson Cancer Center Pathology Core of the University of Pennsylvania. Sections were subjected to immunohistochemistry using antibodies against TRE17 or MMP-9 (R&D, AF909).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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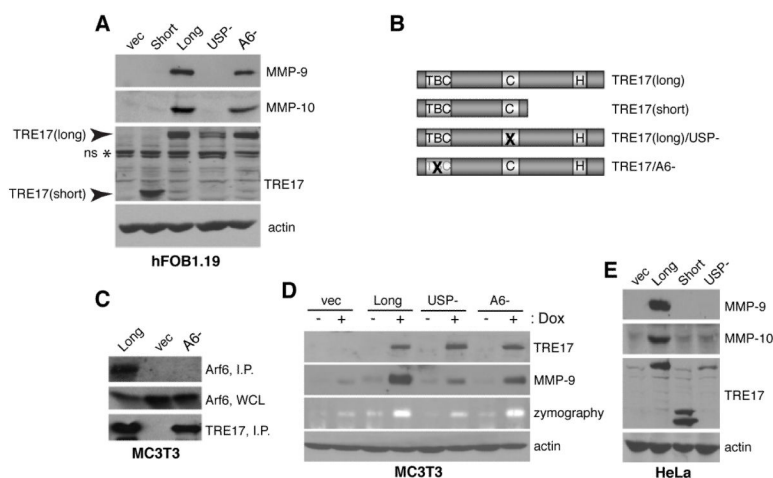
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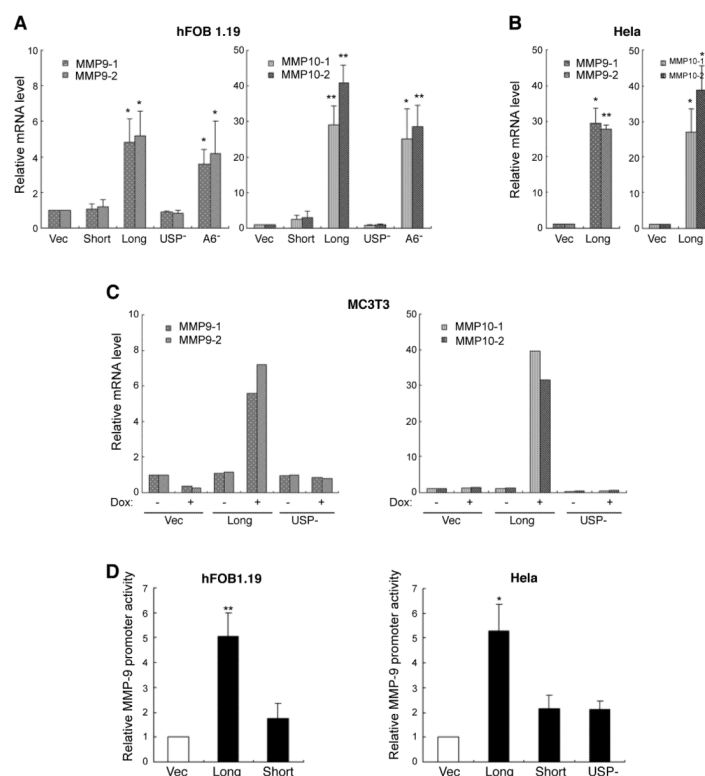
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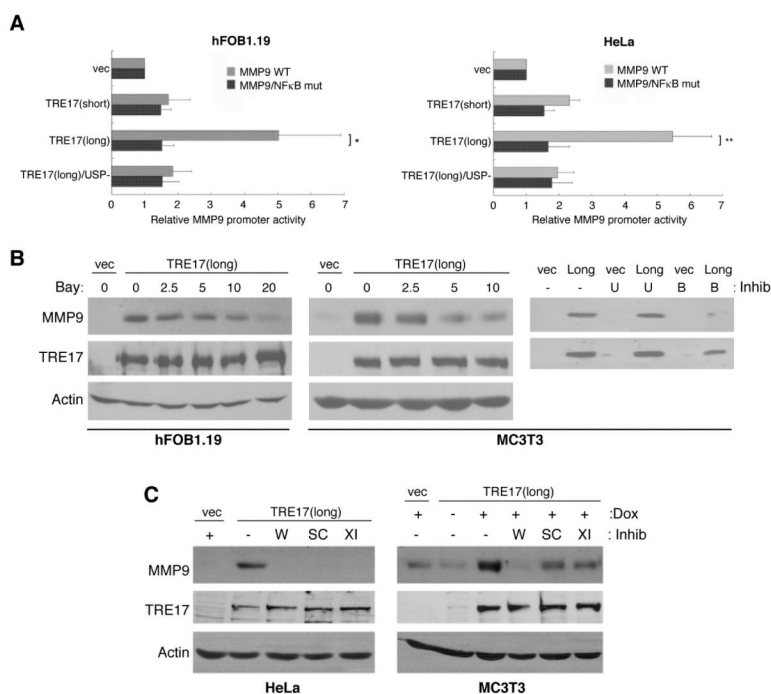


**Figure 1. TRE17 induces secretion of MMP-9 and MMP-10 in a USP-dependent manner**  
**(A)** hFOB1.19 human fetal osteoblasts were transfected with the indicated HA-tagged TRE17 constructs, then serum-starved. MMP-9 and MMP-10 in the CM were concentrated as described in Experimental Procedures, and detected by immunoblotting. Whole cell lysates were blotted for TRE17 (arrowheads indicate migration of TRE17(long) and TRE17(short) isoforms; asterisk denotes non-specific (ns) band recognized by antibody), and actin as a loading control. **(B)** Domain structure of TRE17 alleles. TBC, TBC domain; C and H, cysteine and histidine subdomains of the USP domain. TRE17(long)/USP- and TRE17/A6- harbor point mutations in the indicated domains, as detailed in Experimental Procedures. **(C)** MC3T3 osteoblasts stably expressing HA-tagged TRE17(long) or TRE17(A6-) in a doxycycline (dox)-inducible manner were treated with dox (2  $\mu$ g/ml) for 24 hrs. Cell extracts were immunoprecipitated with anti-HA, then blotted with anti-Arf6 (top panel) or anti-TRE17 (bottom panel). I.P., anti-HA immunoprecipitate; WCL, whole cell lysate. **(D)** Stable MC3T3 cell lines expressing the indicated TRE17 alleles were grown with or without dox for 24 hrs, then starved for 24 hrs in the continued absence or presence of dox. MMP-9 was purified from the CM then subjected to blotting, or zymography to detect gelatinolytic activity. **(E)** HeLa cells were transfected as indicated, and accumulation of MMP-9 and MMP-10 in the CM was monitored as in (A).



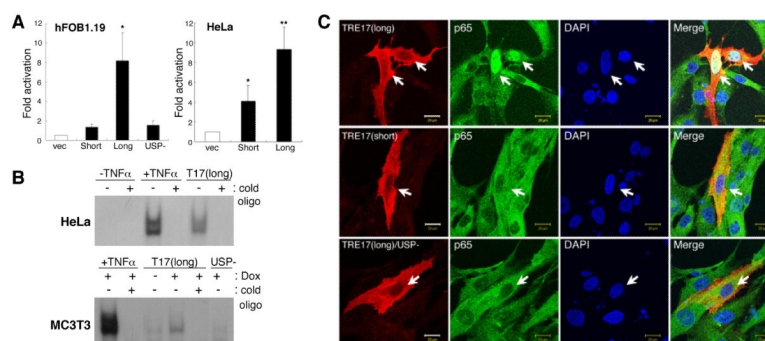
**Figure 2. TRE17 induces MMP expression on a transcriptional level**

(A) hFOB1.19 or (B) HeLa cells were transfected, then serum-starved for 24-48 hours. MC3T3-derived cell lines (C) were treated with or without dox for 24 hrs, then starved for an additional 24 hrs. For all three cell systems RNA was isolated, and RT-qPCR was performed to monitor MMP-9 and MMP-10 mRNA, using two independent primer pairs for each (denoted MMP9-1 and -2, and MMP10-1 and -2), normalizing against GAPDH. For hFOB1.19 and HeLa cells, data represent the mean  $\pm$  SD of three experiments. Samples with statistically significant differences from vector control cells are indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). (D) hFOB1.19 or HeLa cells were co-transfected with the indicated TRE17 constructs and a luciferase reporter driven a fragment of the human MMP-9 promoter encoding base pairs -674 to +3 relative to the transcription start site. Luciferase assays were performed; data represent the mean  $\pm$  SD of 4 experiments. Samples with statistically significant differences from vector control cells are indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).



**Figure 3. NFκB is required for induction of MMP-9 by TRE17**

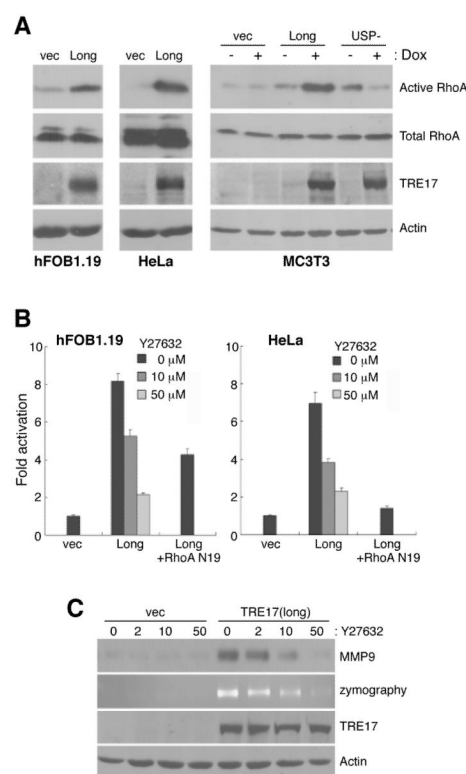
**A)** hFOB1.19 or HeLa cells were transfected with the indicated TRE17 constructs and either a luciferase reporter driven by the WT MMP-9 promoter, or a mutant in which the NFκB response element was ablated. Luciferase assays were performed; data represent the mean  $\pm$  SD of 4 experiments (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). **(B)** Left, hFOB1.19 were transfected as indicated, then starved for 24 hrs. Middle, control or TRE17(long)/MC3T3 cells were treated with dox, then starved in the presence of dox. Bay11-7082 was added at the indicated micromolar concentration during starvation. Right, control or TRE17(long)/MC3T3 cells were treated with Bay11-7082 (B, 10  $\mu$ M) or U0126 (U, 50  $\mu$ M) where indicated. For all three, MMP-9 in the CM was detected; whole cell lysates were blotted with TRE17 and actin antibodies. **(C)** MMP-9 was assayed as in (B), except the IKK inhibitors wedelolactone (W, 50  $\mu$ M), SC514 (SC, 100  $\mu$ M) or IKK Inhibitor XI (XI, 20  $\mu$ M) were used where indicated.



**Figure 4. TRE17(long) activates NFκB in a USP-dependent manner**

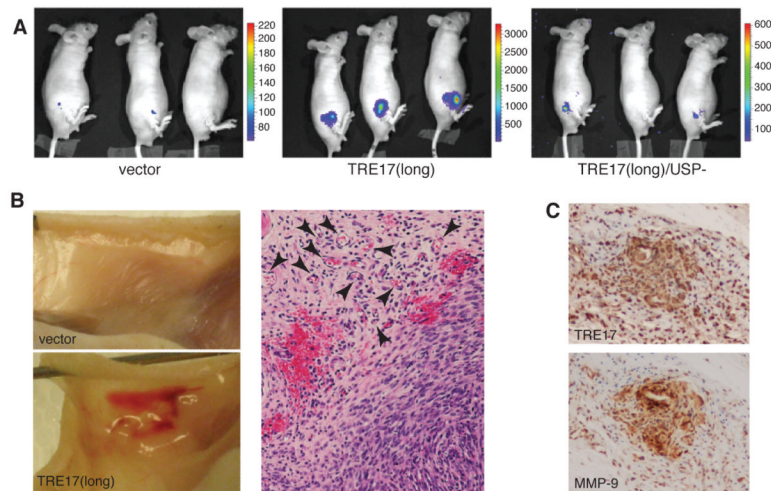
(A) hFOB1.19 or HeLa cells were co-transfected with the indicated TRE17 construct and a luciferase reporter driven by tandem NFκB response elements. Cells were starved and luciferase assays were performed. Data represent the mean  $\pm$  SD of four independent experiments (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). (B) EMSAs were performed in transiently transfected HeLa (top), or MC3T3 lines expressing vector, TRE17(long), or TRE17(long)/USP<sup>-</sup> (bottom). As a positive control, vector control cells were treated with TNFα (10 ng/ml, 30 minutes) where indicated. (C) hFOB1.19 cells were transfected with the indicated TRE17 construct, starved for 24 hr, then stained with anti-HA, anti-p65, and DAPI. Arrows highlight transfected cells. Scale bar, 20 μm.





**Figure 5. RhoA and ROCK partially mediate activation of NF $\kappa$ B by TRE17**

(A) RhoA activity was measured in transiently transfected hFOB1.19 (left), HeLa (middle), and MC3T3 cell lines (right). Cell extracts were subjected to pulldowns using a GST fusion of the RhoA-GTP-binding domain (RBD) of Rhotekin, followed by blotting with anti-RhoA. Active RhoA, RBD pulldowns; total RhoA, whole cell lysates. (B) hFOB1.19 or HeLa were transfected with NF $\kappa$ B-luciferase and TRE17(long), in the absence or presence of dominant negative RhoA (RhoAN19) or Y27632, and subjected to luciferase assays. (C) Control or TRE17(long)/MC3T3 cells were treated with dox for 24 hrs, then starved with dox and the indicated concentration of Y27632 ( $\mu$ M) for 24 hrs. MMP-9 in the CM was detected by immunoblotting and zymography. Whole cell extracts were blotted to confirm TRE17 and actin levels.



**Figure 6. TRE17(long) induces formation of tumors that recapitulate multiple features of ABC in a USP-dependent manner**

(A) *In vivo* bioluminescence imaging of mice injected with MC3T3 cell lines expressing TRE17(long), TRE17(long)/USP-, or luciferase alone. (B) Left, macroscopic images of tumor and injection site of mice injected with vector or TRE17(long)-expressing MC3T3. Right, H&E stain of TRE17(long) tumor section. Arrowheads highlight vascular elements. (C) Immunohistochemistry was performed on serial sections of TRE17(long)-induced tumor using antibodies against TRE17 and MMP-9. Nuclear staining for TRE17 is non-specific; only central cells exhibit specific cytoplasmic staining of TRE17.